ANNUAL REPORT

for

NASA Contract NAS 9-15566

Purification and Cultivation of Human

Pituitary Growth Hormone Secreting Cells

by

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I. *General Introduction*

Our efforts are directed toward maintenance of actively secreting human pituitary growth hormone cells (somatotrophs) in vitro. The production of human growth hormone (hGH) by this means would be of benefit for the treatment of certain human hypopituitary diseases such as dwarfism. Since the chemical nature of the factor(s) responsible for stimulating the release of hGH from the somatotroph is totally unknown, one of our primary approaches has been the testing of agents which may logically be expected to increase hGH release. This report summarizes our progress towards this goal. It also describes results from preliminary experiments dealing with electrophoresis of pituitary cells for the purposes of somatotroph separation. Results from these (and ongoing) experiments will eventually provide the data required to evaluate the need and potential use of space electrophoresis for the purpose of obtaining pure somatotrophs which produce growth hormone in culture.

II. *Tissue Procurement*

Shortly after initiation of this contract, we began setting up procedures for the procurement of human pituitary tissue; either biopsy or post-mortem specimens. The following pathologists and neurosurgeons are collaborating on this project:

Dr. Andrew Dekker, University of Pittsburgh, Pittsburgh, PA

Dr. Olof Pearson, Case Western Reserve, Cleveland, OH

Dr. Robert Page, Hershey Medical Center, Hershey, PA

Dr. Thomas Pretlow, University of Alabama, Birmingham, AL

Dr. Bruce Northrup, Thomas Jefferson Medical School, Philadelphia, PA

In all cases, these individuals mince the tissue into $\sim$ 1-3 mm^3 fragments and send them to Penn State in bottles containing 50 ml of sterile medium 199 containing 5% horse serum and pen-strep (100 μ/ml). These bottles, which are shipped at ambient temperature, usually arrive in our laboratory within
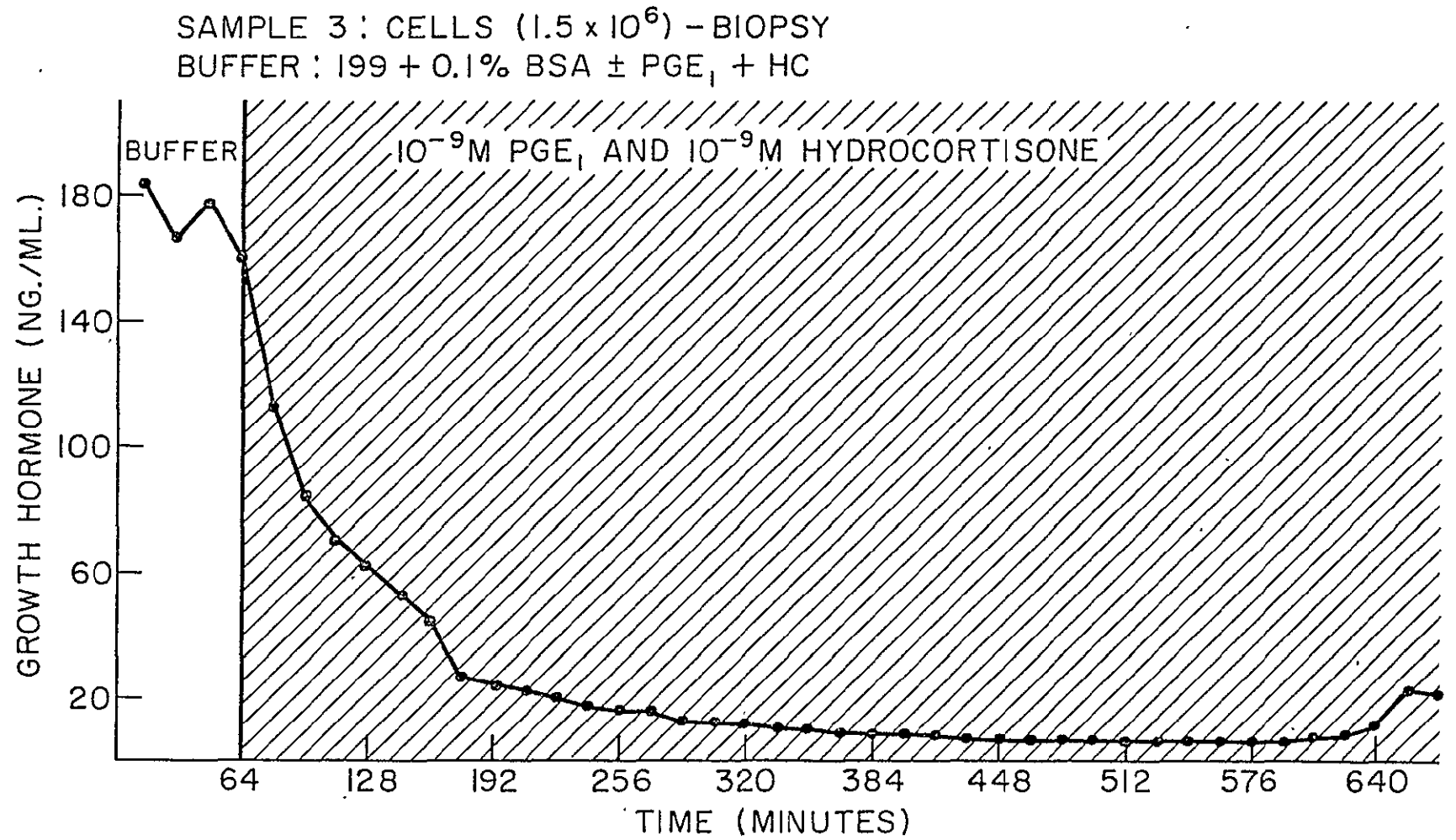
48 hours after removal of the gland. Our samples from Pittsburgh usually arrive within 18 hrs. As of 4/30/79, i.e., one year after initiation of this contract, we had received 71 human pituitary glands, 9 of which were biopsy (i.e., surgical) specimens. Of the 62 post-mortem glands, 35 came from Dr. Pretlow and 27 from Dr. Dekker. Careful records are kept concerning relevant patient information. In most cases this can be found on the figures in this report.

III. *"Cultivation" of Human Pituitary Cells/Tissue. Summary of the first 6 months work (5/1/78-10/31/78).*

Since this material was detailed in the mid-term report to NASA, it is only briefly reconsidered in this section.

We developed 3 different enzymatic procedures for tissue dissociation, the most satisfactory of which was the one using a solution containing Medium 199 + 0.5% BSA + 0.3% collagenase type I. Cell yields ranged from $0.3-3.1 \times 10^6$ single cells/gland with viability $\sim 85\%$. Staining of these preparations by Herlant's tetrachrome procedure indicated that most of the cell types were readily identifiable, including the somatotroph. The cell preparations were subsequently placed in Biogel columns and perfused with agents which we had reason to believe, on the basis of our work with <u>rat</u> somatotrophs, would release appreciable quantities of stored intracellular hormone into the perfusate. These agents included: dibutyryl cyclic AMP (cAMP), prostaglandin E_1 (PGE_1) and hydrocortisone (HC). The results in Fig. 1 are representative of those obtained in several other experiments. The rapid decline in hormone output immediately after placing the cells/tissue in the column is routinely encountered in this experimental protocol and probably can be attributed to tissue manipulation and equilibration with the perfusing buffer. It is obvious from Fig. 1 that HC and PGE_1, at physiological concentrations (10^{-9} M), had no effect on release of hGH. Similarily, perfusion with cAMP (1-10 mM), was equally ineffective in promoting hormone release (data not shown).

Figure 1

SAMPLE 3 : CELLS (1.5 x 10^6) – BIOPSY
BUFFER : 199 + 0.1% BSA ± PGE$_1$ + HC

BUFFER

10^{-9}M PGE$_1$ AND 10^{-9}M HYDROCORTISONE

GROWTH HORMONE (NG./ML.)

180

140

100

60

20

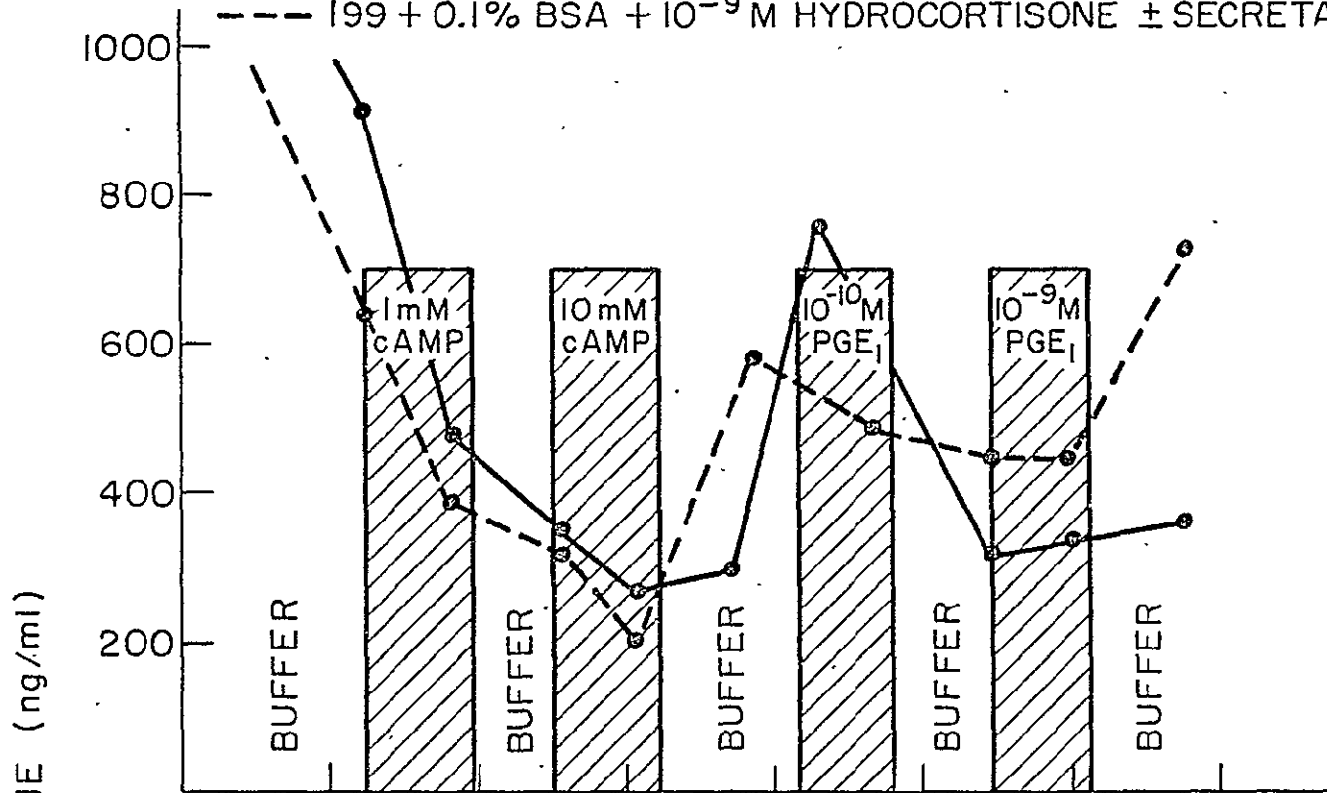64   128   192   256   320   384   448   512   576   640

TIME (MINUTES)

SAMPLE I : PIECES

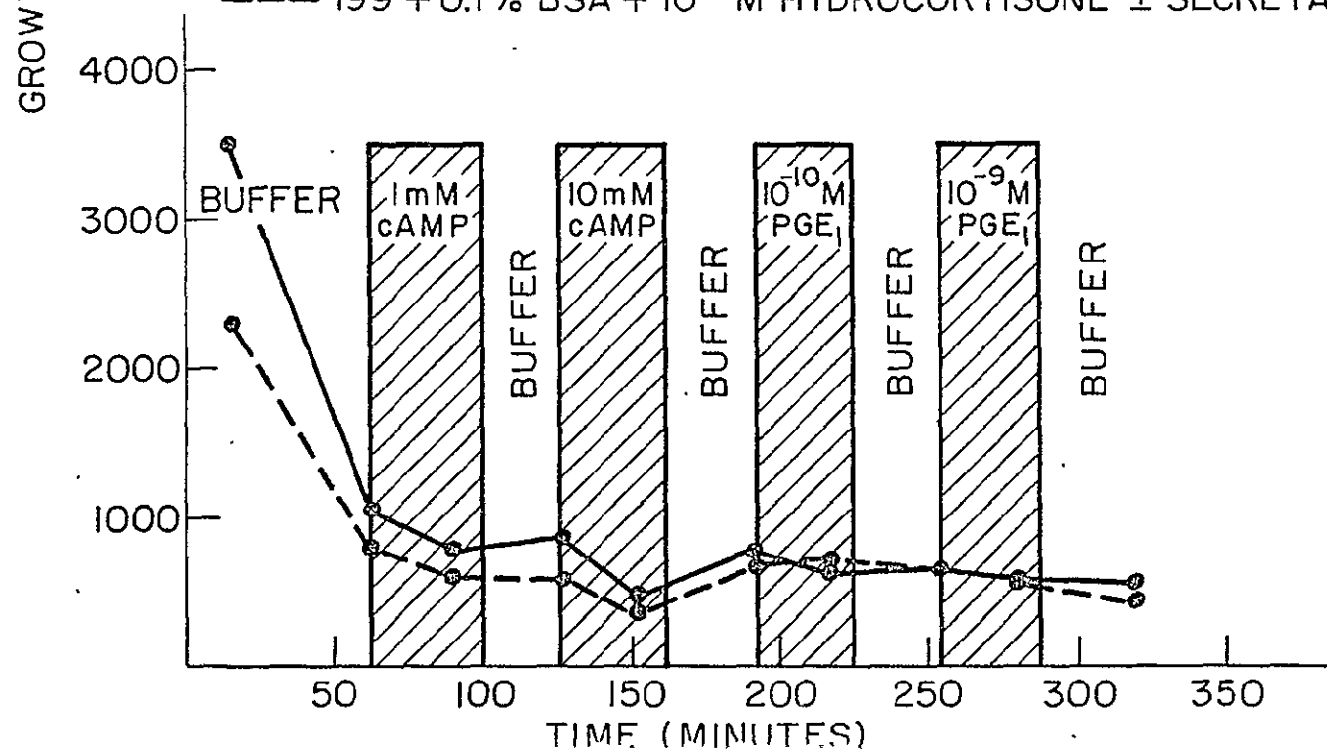—— 199 + 0.1% BSA ± SECRETAGOGUE

----- 199 + 0.1% BSA + 10^{-9} M HYDROCORTISONE ± SECRETAGOGUE

SAMPLE IO : PIECES

—— 199 + 0.1% BSA ± SECRETAGOGUE

----- 199 + 0.1% BSA + 10^{-9}M HYDROCORTISONE ± SECRETAGOGUE

To examine the possibility that the somatotrophs were rendered un-responsive due to enzyme treatment, we also did an experimental series in which 1-2 mm^3 pieces of tissue were placed in the glass microchromatography column. As shown in Fig. 2, neither cAMP, PGE$_1$, nor HC had any appreciable effect on hormone release.

In sum, these results suggested to us the very real possibility that the human and rat somatotroph are two very different "creatures." Accordingly, we expanded our search for secretagogues in the second half of the first years' effort. These results are considered in detail below.

IV. _"Cultivation" of Human Pituitary Cells/Tissue. Summary of the second 6 months work (11/1/78-4/30/79)._

A decision was made to expand the effort in favor of using pieces of tissue in the microchromatography column. There were two reasons for this decision; first, the number of single cells which we could obtain out of some post-mortem samples (especially those > 7 hrs) was often only ∿ 300,000 cells; second, the culture data obtained when these low cell numbers were used often resulted in correspondingly low hGH levels (< 20 ng/ml). By comparison, hGH levels from tissue pieces were usually appreciable (300-800 ng/ml).

A decision was also made at this time to search for new agents which might stimulate hGH release since our previous data had indicated that those agents which stimulated hormone release in the rat system (cAMP, HC, PGE$_1$) were not effective in the human system (Figs. 1 and 2). Accordingly, we tested effects of ascites fluid, TRH, cholera toxin and hypothalamus on release of hGH from tissue pieces.

ASCITES FLUID

It is becoming clear that various kinds of tumor cells and tumor fluids are capable of making certain hormones and/or hormone releasing factors. On

this basis we decided to test ascitic fluid taken from rats bearing the ascites form of the 13762 mammary adenocarcinoma for growth hormone releasing factor activity. This material was chosen since we maintain this tumor line in our laboratory. The results from two separate experiments using ascites fluid (AF), on human pituitary pieces maintained according to the column protocol are shown in Fig. 3. In the first experiment, brief (8') pulses with increasing concentrations of AF indicated that GH release was significantly stimulated at the 50% dose, and more so when pure AF was used. In the second experiment, a continuous pulse with 4% AF for ∿ 16 hrs resulted in a spike of GH release for the first 4 hrs followed by a gradual decline toward basal levels. These experiments, taken together, offer good evidence for the presence of GRF-activity in AF. This is a useful and exciting clue. In the second year of the contract we propose to do more experiments along this line.

THYROTROPIN RELEASING HORMONE (TRH)

It is well documented that this synthetic tripeptide stimulates release of TSH and prolactin. In isolated cases it has also been reported to stimulate GH release. Our experiences with this agent are shown in Fig. 4. In the "short pulse mode" (left panel) evidence for GRF activity is seen although it is sporadic and apparently not related to dose. A continuous pulse of the tripeptide did not result in stimulation of GH release. On the basis of these results and those in the recent literature, we therefore conclude that TRH has, at best, only weak GRF activity. We will not use this agent alone in future experiments, but we may use it in combination with other agents (see later).

CHOLERA ENTEROTOXIN (CT)

This glycoprotein (84,000 MW) has been reported to activate a number of endocrine cells. Apparently it does so by binding to a receptor on the cell surface. There is evidence to suggest that this receptor, which may be
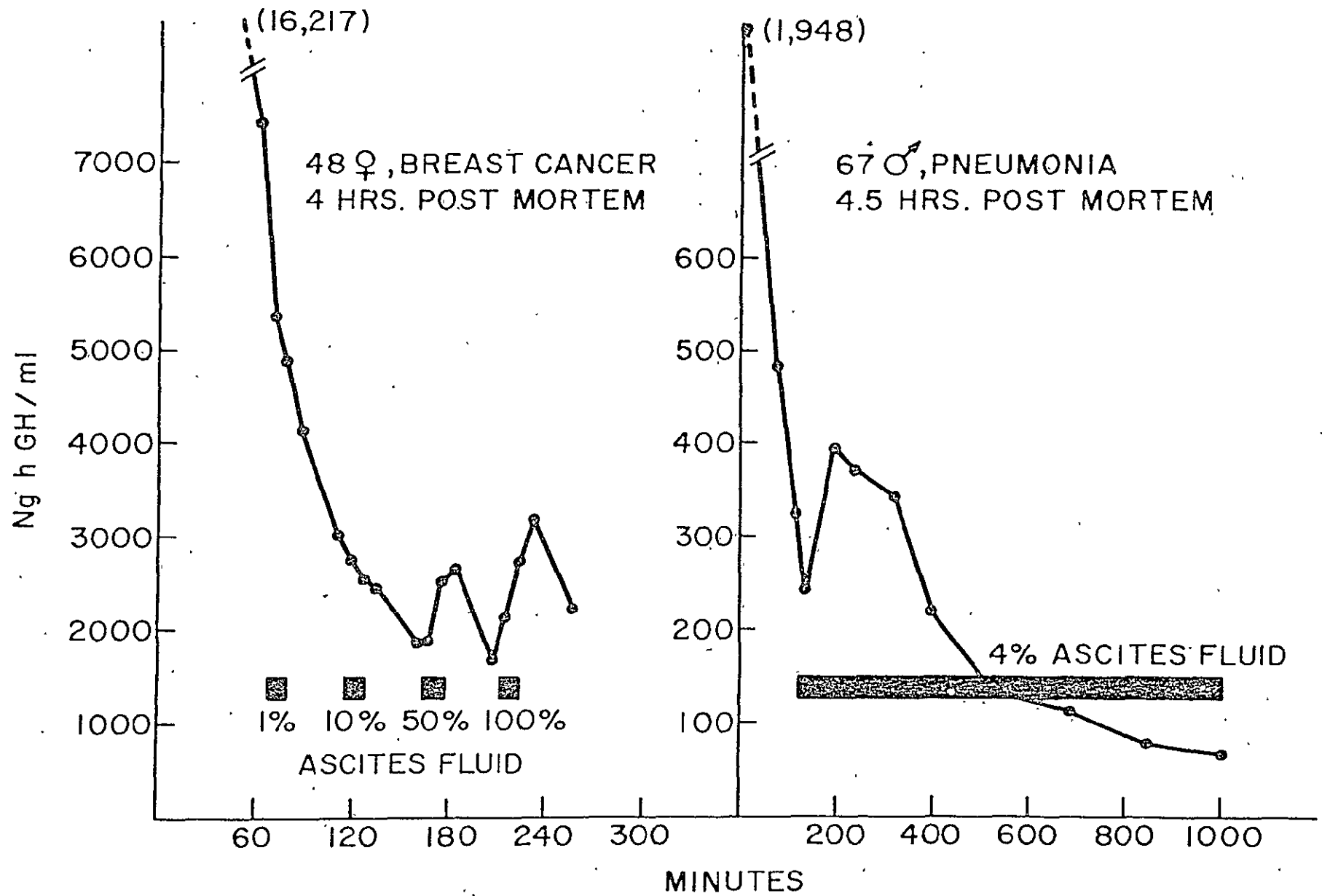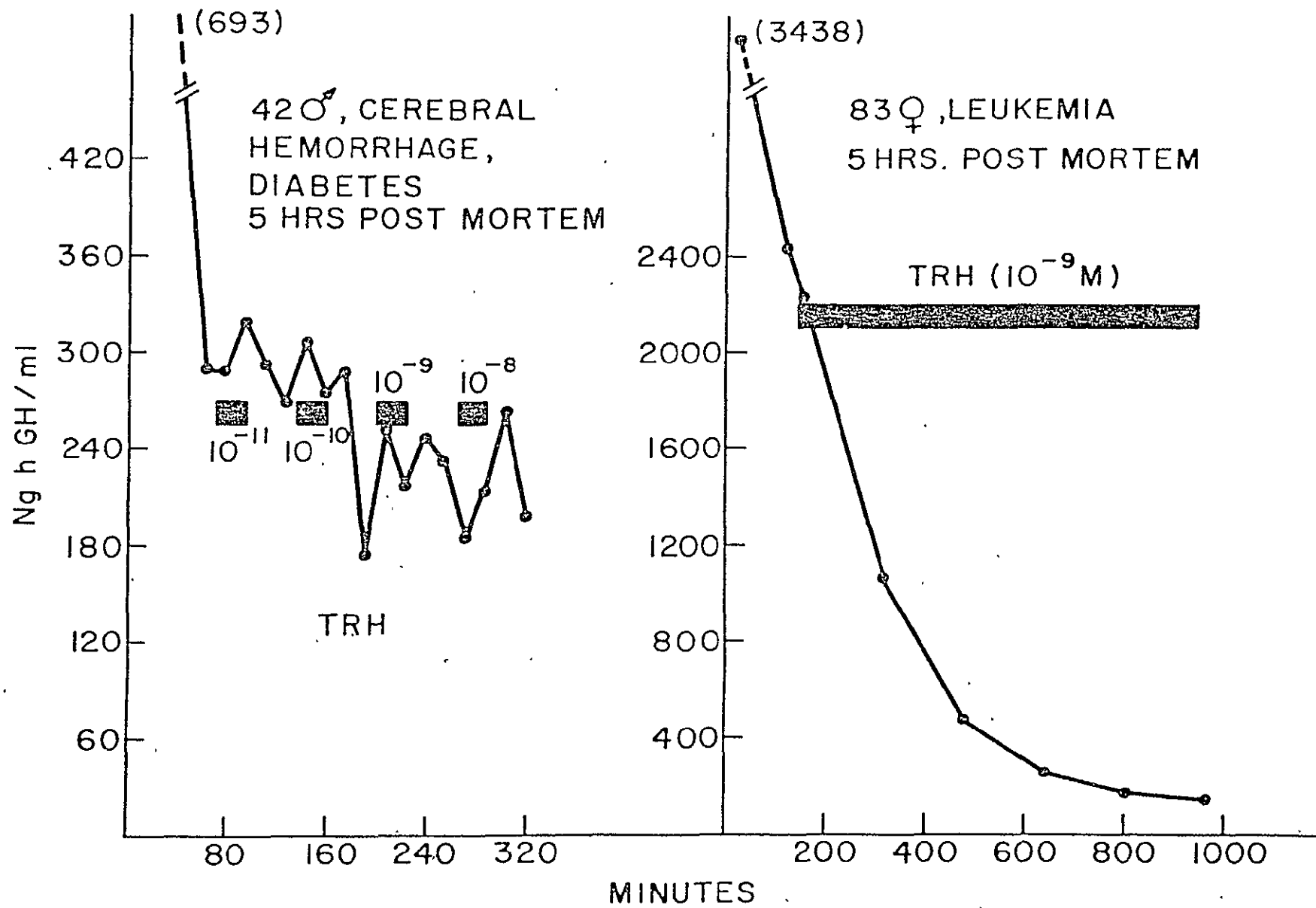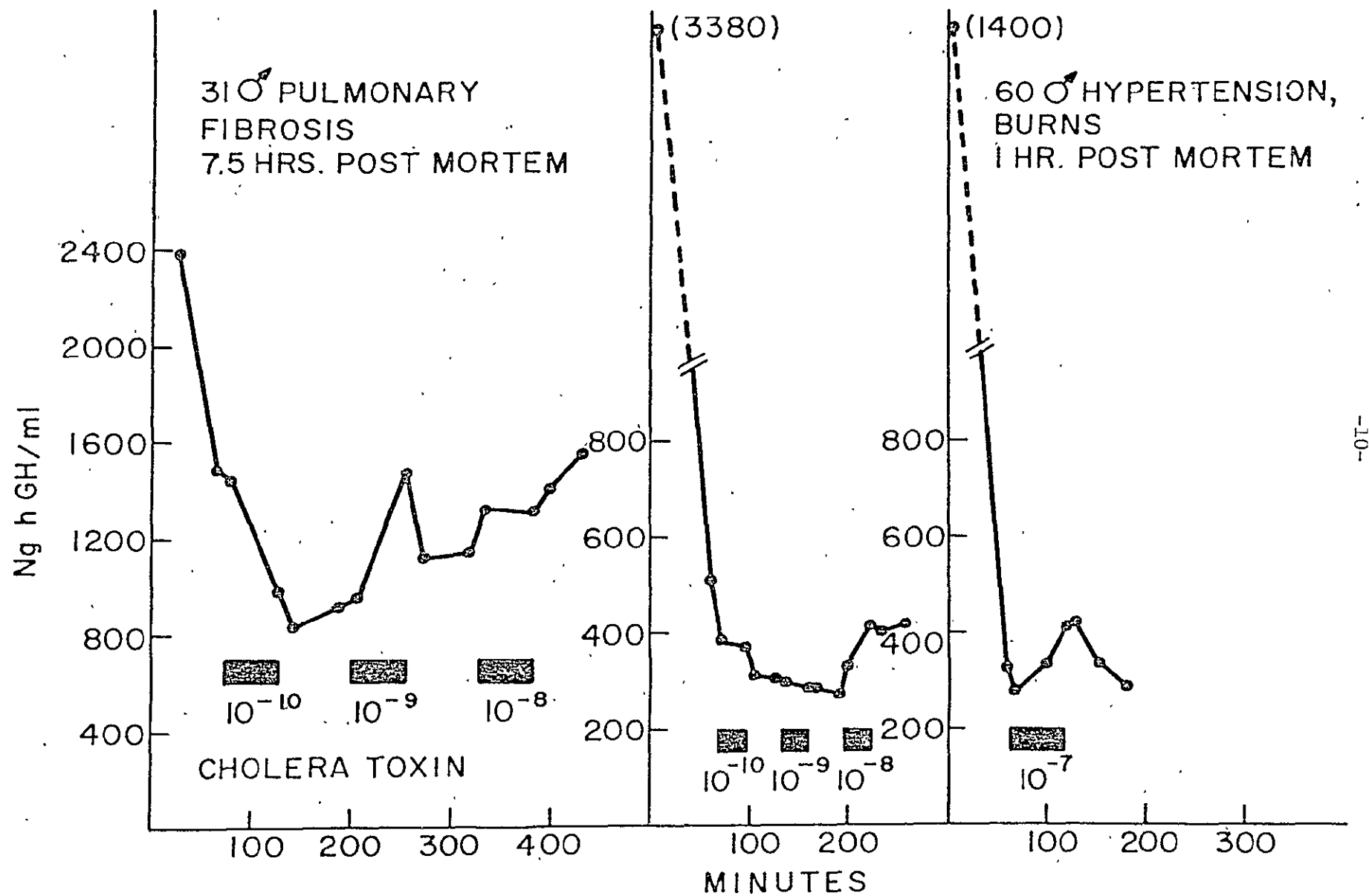
Figure 3

Figure 3

Figure 4

a GM_1 ganglioside, is linked to adenylate cyclase--the enzyme which catalyzes conversion of intracellular ATP to cAMP. The reader may recall from our 6 month report that dibutyryl cyclic AMP did not stimulate hGH release from tissue pieces. However, since cAMP has been shown to play a key role in the mechanism of action of many hormones, we decided to perform several experiments utilizing CT as the potential secretagogue. Our first protocols called for CT administration in the "pulse mode." As seen in Fig. 5, 45-60' pulses with CT ($10^{-9}-10^{-7}$ M) resulted in rather marked elevations of hGH release in each of 3 separate experiments. From the data in Fig. 5, it appears that the minimal effective dose may be somewhere in the range of $10^{-8}-10^{-9}$ M.

In a second type of experimental protocol, a single dose of CT (10^{-9} M) was continuously perfused over the tissue pieces overnight. The results from 2 such experiments (see Fig. 6) document that CT does indeed sustain hGH in vitro over prolonged time periods. The top curve to Fig. 6 is especially dramatic in this regard. The kinetics of release from the bottom curves in Fig. 6, while not as dramatic, are nonetheless noteworthy since hormone release is sustained (albeit at low level) overnight. The reader will have also recognized that the GH levels coming out of the gland at the beginning of the experiment are extremely variable. It has been our experience that the magnitude of these initial levels, while uncontrollable experimentally, clearly affect the final GH levels obtained in any given experiment.

Finally, in a third experimental protocol involving CT, we tested the effectiveness of this agent on dispersed single human pituitary cells (obtained by collagenase digestion). In some cases we pooled 2 human pituitary samples in an effort to obtain sufficient cell numbers for the biogel column. The data from 2 such experiments are shown in Fig. 7. In the first experiment, CT, in combination with PGE_1, HC and TRH (all at 10^{-9} M) was ineffective in promoting release of hGH from biopsy pituitary. In a similar experiment (middle

Figure 5

(2814)

900

800

700

600

Ng hGH/ml

500 △——△ 59 ♀ ; CHRONIC HEART FAILURE ;
(INSULIN DEPENDANT
DIABETIC): 3.5 HRS. POST
MORTEM

400

⊙——⊙ 63 ♂ ; HEART FAILURE (EPINEPHRINE +
300 DOPAMINE PRIOR TO DEATH):
— HRS POST MORTEM

200

100

10^{-9} M CHOLERA TOXIN

200   400   600   800   1000   1200

MINUTES

Figure 7

41 ♀ – BIOPSY
BREAST CANCER
(740,000 CELLS)

PGE$_1$ + HC + TRH
+ CHOLERA TOXIN
(10^{-9}M)

71 ♂; ARTHRITIS
9 HRS POST MORTEM
+
60 ♂; MELANOMA
2 HRS POST MORTEM
(3.6 x 10^6 CELLS)

PGE$_1$ + HC + TRH
+ CHOLERA TOXIN(10^{-9}M)

59 ♂, HEART FAILURE
3 HRS POST MORTEM
+
47 ♀, MYELOMA
6 HRS POST MORTEM
(600,000 CELLS)

CHOLERA TOXIN (10^{-9}M)

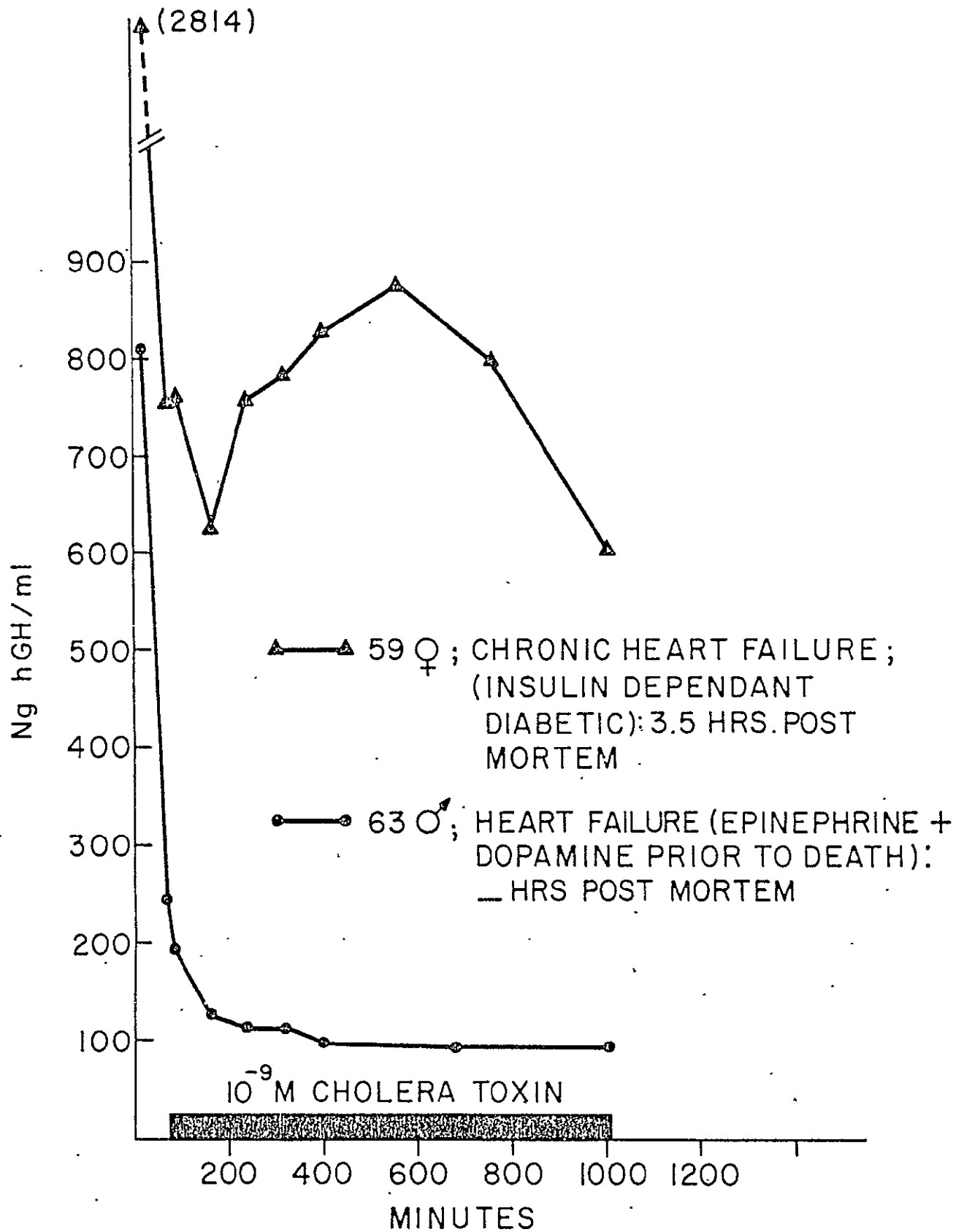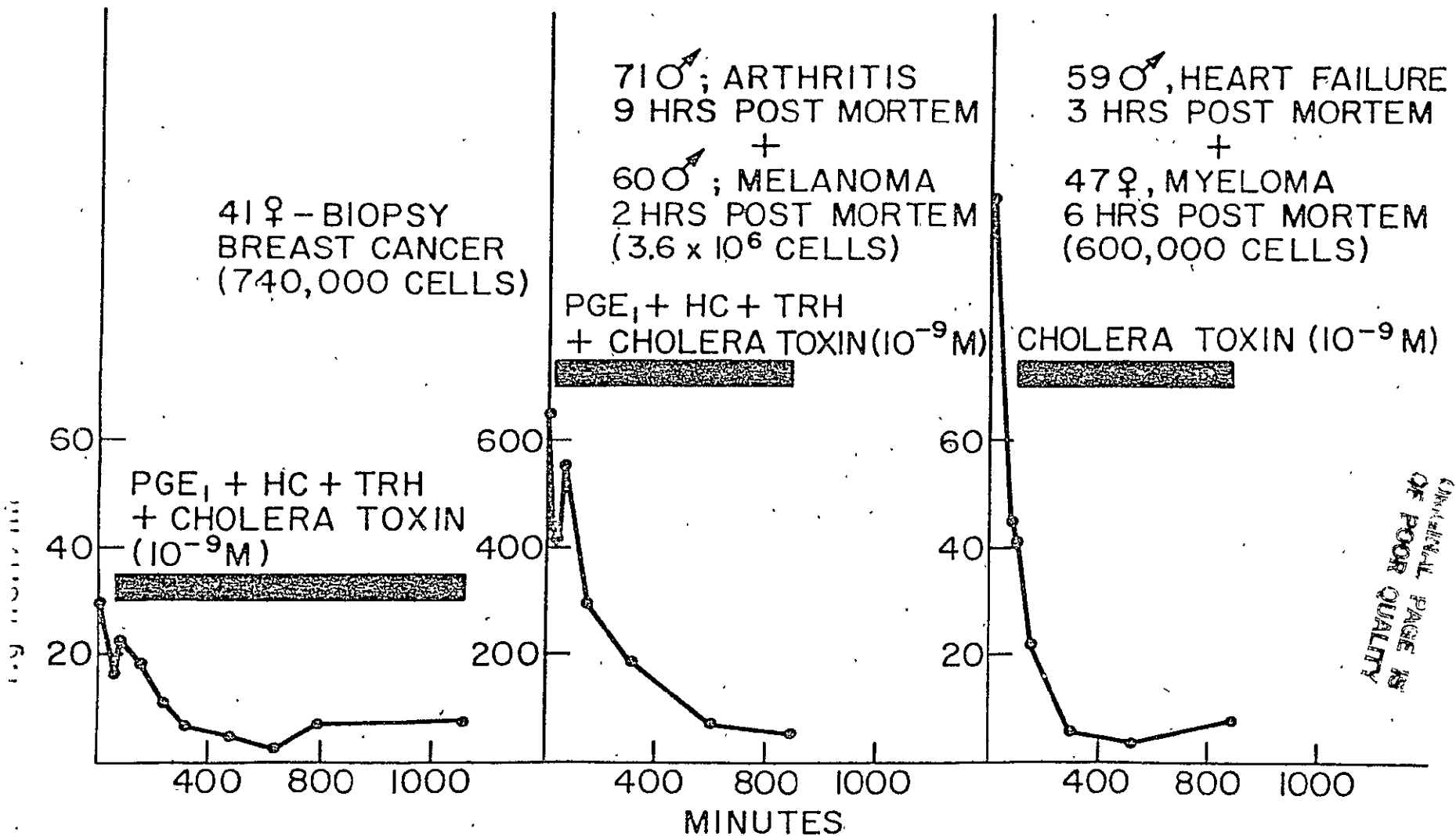MINUTES

panel, Fig. 7) with cells from combined pituitaries, negative results were also obtained.

We postulate that the reason the CT is effective on tissue pieces (Figs. 5 and 6), but not on dispersed cells (Fig. 7) is that the collagenase preparation effectively strips CT receptors from the cell membrane. This conclusion has important implications for electrophoresis of CT-responsive pituitary cells. In this specific regard it would be interesting to see if collagenase-dispersed pituitary cells, maintained in culture for 1-3 days, become responsive to CT. If so, it would provide good evidence for CT receptor regeneration in vitro. Such experiments are planned for the coming year. In the coming year we also plan on seeing how long the CT effect persists in the pieces-perfusion mode.

RAT HYPOTHALAMUS

It is well documented that the known hypothalamic releasing hormones, viz. TRH, LHRH and somatotstain are not species specific. Although the structure of growth hormone releasing factor (GRF) is completely unknown, we reasoned that GRF in the rat hypothalamus had the potential of being able to stimulate GH release from the human gland. Of course the situation is complicated by the presence of somatostatin (SIF) in the hypothalamus. Nevertheless, we decided to carry out an experimental series in which rat hypothalamus was "linked" to human pituitary pieces. This was accomplished in 2 ways; either by halves of hypothalamus in one column and having its eluant "drain" into a second column containing human pituitary or by placing hypothalamus tissue above the pituitary tissue in the same column. In general, the results obtained thus far seem relatively independent of the configuration used.

The 5 experiments depicted in Fig. 8 document the kind of result obtained when rat hypothalamus is linked to human pituitary. In the first panel, a clear and dramatic response was obtained when effluent from the hypothalamus column (prepared from 7 Fisher 344 ♀ rats 42 days old) passed through pieces

of human pituitary removed at surgery for pallitative treatment of breast cancer. Note an obvious rise in GH output was observed. Interestingly, this output was not well correlated with the timing of the hypothalamus pulse. In the second panel an experiment is shown where this identical protocol had no effect on GH output. In the third experiment a small, but probably significant rise in GH output occurred after the hypothalamus pulse. The results from the fourth experiment clearly show that brief (10 min) pulses of hypothalamus result in episodic bursts of hormone release. In the fifth experiment (right hand panel, Fig. 8), hypothalami from 12 rats were homogenized in cold 0.1 N HCl, centrifuged, and the clear supernatant fraction added to the pituitary column as a brief pulse (after neutralization). The results show that an active GRF was present in this extract used at a concentration of 0.5 hypothalamic equivalents. Essentially negative responses were obtained with lower doses of the extract.

In summary, the results from Fig. 8 offer fairly good evidence for the existence of a GRF in rat hypothalamus which can stimulate hGH secretion. However, the variability in the results indicated to us that additional ways of stimulating GRF from the hypothalamic tissue were required.

RAT HYPOTHALAMUS ± EPINEPHRINE ± PGE$_1$

There are indications in the literature that small neurotransmitter molecules may release the releasing hormones from the hypothalamus. Molecules such as dopamine, serotonin, endorphine, substance P, prostaglandin, epinephrine, norepinephrine, melatonin, etc. have all been considered as possible candidates for such a role. In the first year of our contract we chose to test PGE$_1$ and epinephrine since, from the literature, these agents seemed the most likely candidates for being able to stimulate release of GRF from the hypothalamus. The left panel to Fig. 9 shows the response when Medium 199 containing 0.1% BSA and 10^{-9} M PGE$_1$ first flows through a column containing rat
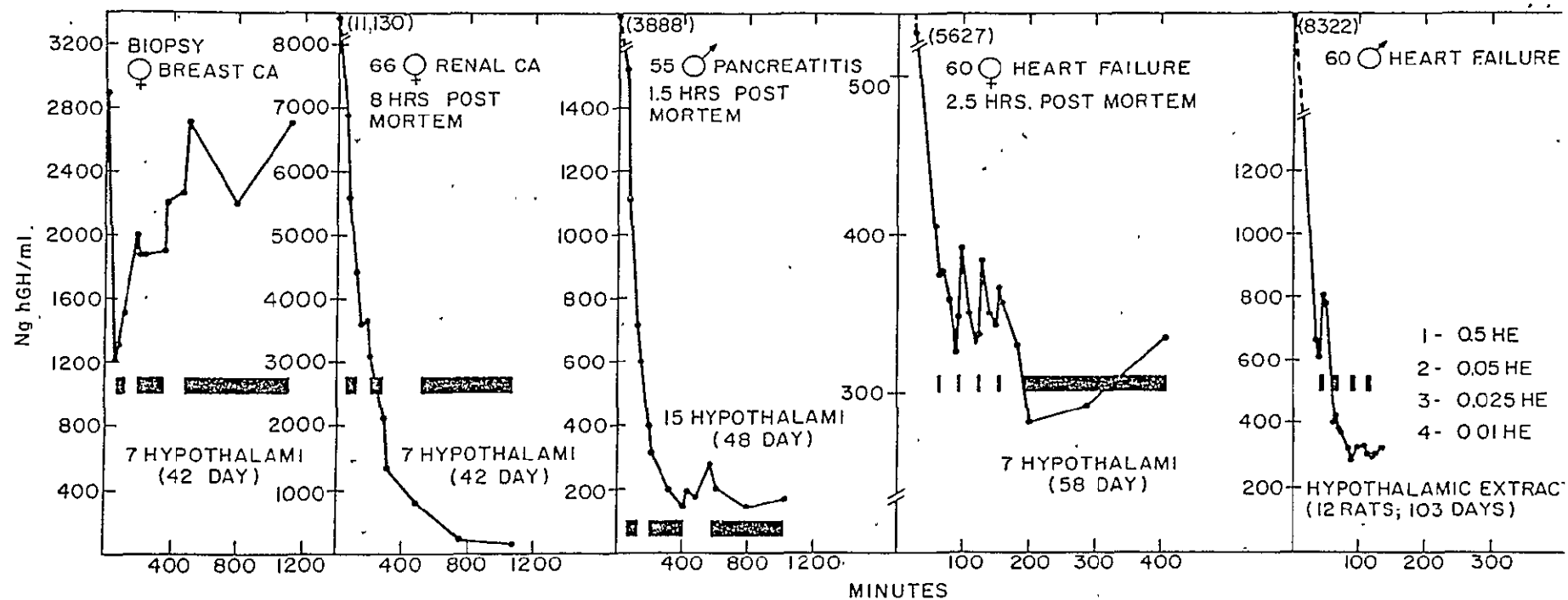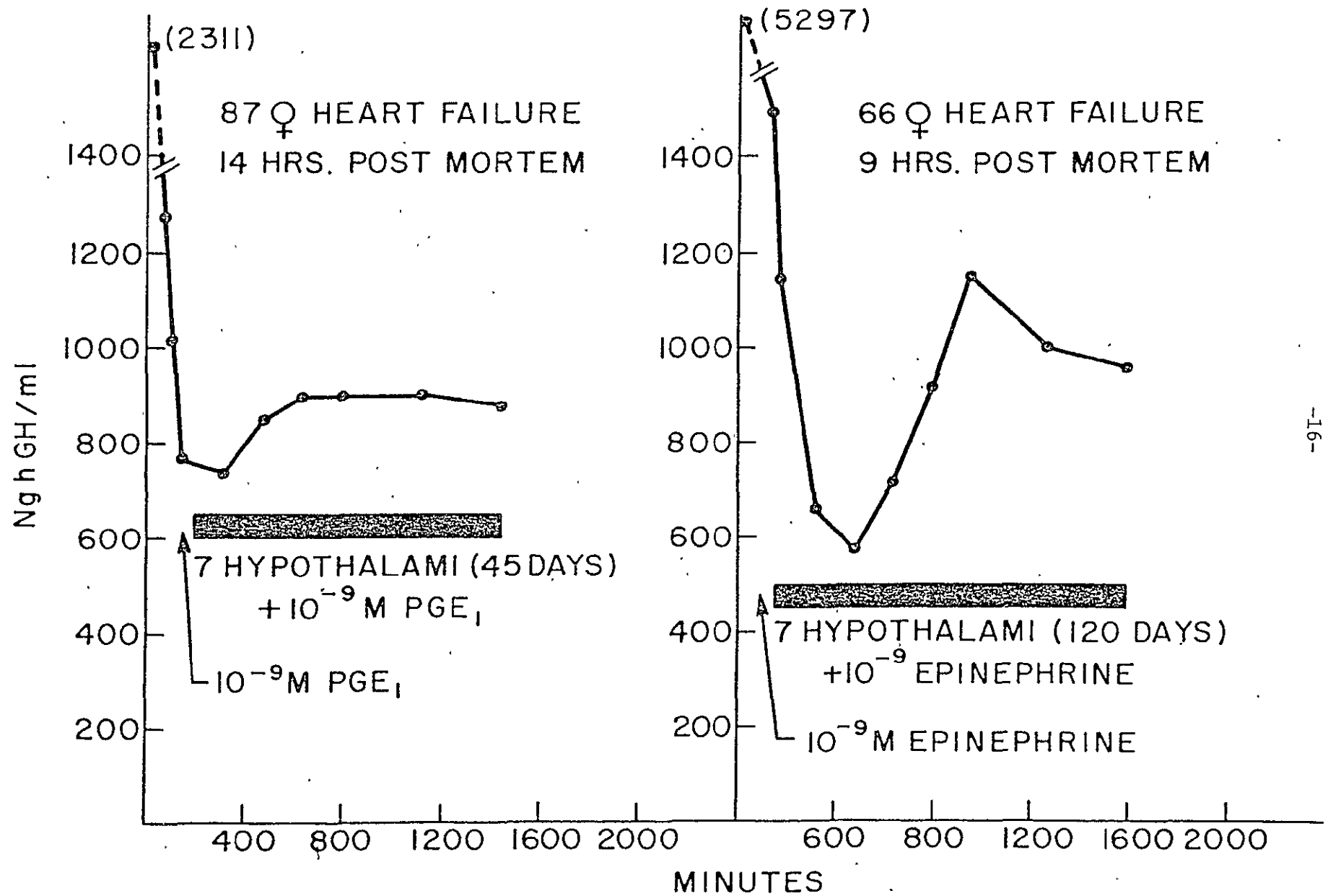
Figure 8

Figure 9

hypothalami prior to flowing through the pituitary column. Growth hormone release falls rapidly prior to perfusion through the hypothalamus column, even though this buffer contained 10^{-9} M PGE$_1$. This results suggests that PGE$_1$, by itself, has no effect on hormone release. A similar conclusion can be drawn from work done earlier in the contract year (e.g., see Fig. 2 of this report). As seen in Fig. 9 (left panel) GH release was stable within 1 hour after "connecting" the hypothalamus column to the pituitary column. Two hours later GH release was elevated and maintained at this higher level throughout the remainder of the experiment.

The right hand panel of Fig. 9 shows an approximately similar result. In this case 10^{-9} M epinephrine was used as the agent to release the GRF. It is interesting that 5-6 hours after pulsing through the hypothalamus column, GH release rose in linear fashion over the next 6 hour period.

The reader will have noticed that the pituitary tissues were 9-14 hrs post-mortem. Considering that 2-3 days are required to ship the tissues to University Park, the results are rather remarkable in that they clearly document the potential usefulness of such tissue for the ultimate "production" of hGH. In this regard morphological analyses of these tissues, upon completion of the experiment, would be useful in terms of assessing tissue viability. Such studies are planned for the second year of the contract.

Finally, it is worthwhile emphasizing that these data, to my knowledge, are among the first to show successful maintenance of actively secreting human pituitary somatotrophs in vitro. Obviously more work is required, but the basic framework of the response seems unequivocally established.

V. *Isoelectric Focusing of Rat Pituitary Cells. Summary of the first 6 months work (5/1/78-10/31/78).*

Since the isoelectric focusing methodology was detailed in the mid-term report, it will not be repeated here.

In the first experimental series we found out that recovery of cells from the electrophoresis column was ∿ 50%, regardless of the temperature, time or pH range of the gradient used in the experiment. From other experiments we noted that this loss was apparently due to the acidic pH of the electrophoresis column. This result thus showed that some pituitary cells could not withstand acidic pH. However, of the 50% of the cells which were recovered we noted a distribution profile which showed that one population of cells consistently focused at pH ∿ 5.25. Other cells were often recovered at pH ∿ 5.6 and 4.9. These patterns (Fig. 10) seemed reasonably independent of temperature (4° vs 15°) and time (60' vs 180'). Histological analysis of the recovered cells had not been done at that time.

VI. _Isoelectric Focusing of Rat Pituitary Cells. Summary of the second 6 months work (11/1/78-4/30/79)._

Quantitative data relating to cell recovery in five isoelectric focusing experiments are given in Table 1. The percentage of cells recovered from the electrophoresis column was extremely variable, ranging 14-86%. Especially interesting was the finding that cell recovery, after sitting in insertion buffer at the same temperature for the same time as in the electrophoresis run, was significantly greater. As before, we interpret this result to indicate that some pituitary cells cannot withstand acidic pH's to which they eventually focus.

The distribution of cells recovered from the first 3 experiments itemized in Table 1 is given in Fig. 11. The cell profiles reveal a major cell peak with tailing edges in each case. Even when the insertion pH was quite high (NP47-pH 6.2) the cells were eventually recovered around fraction 15. Since the cell recovery was quite good in NP-47 (86%), the result suggests that a more neutral insertion pH point is beneficial in terms of pituitary cell stability. Although not presented here, the cytological analyses of the
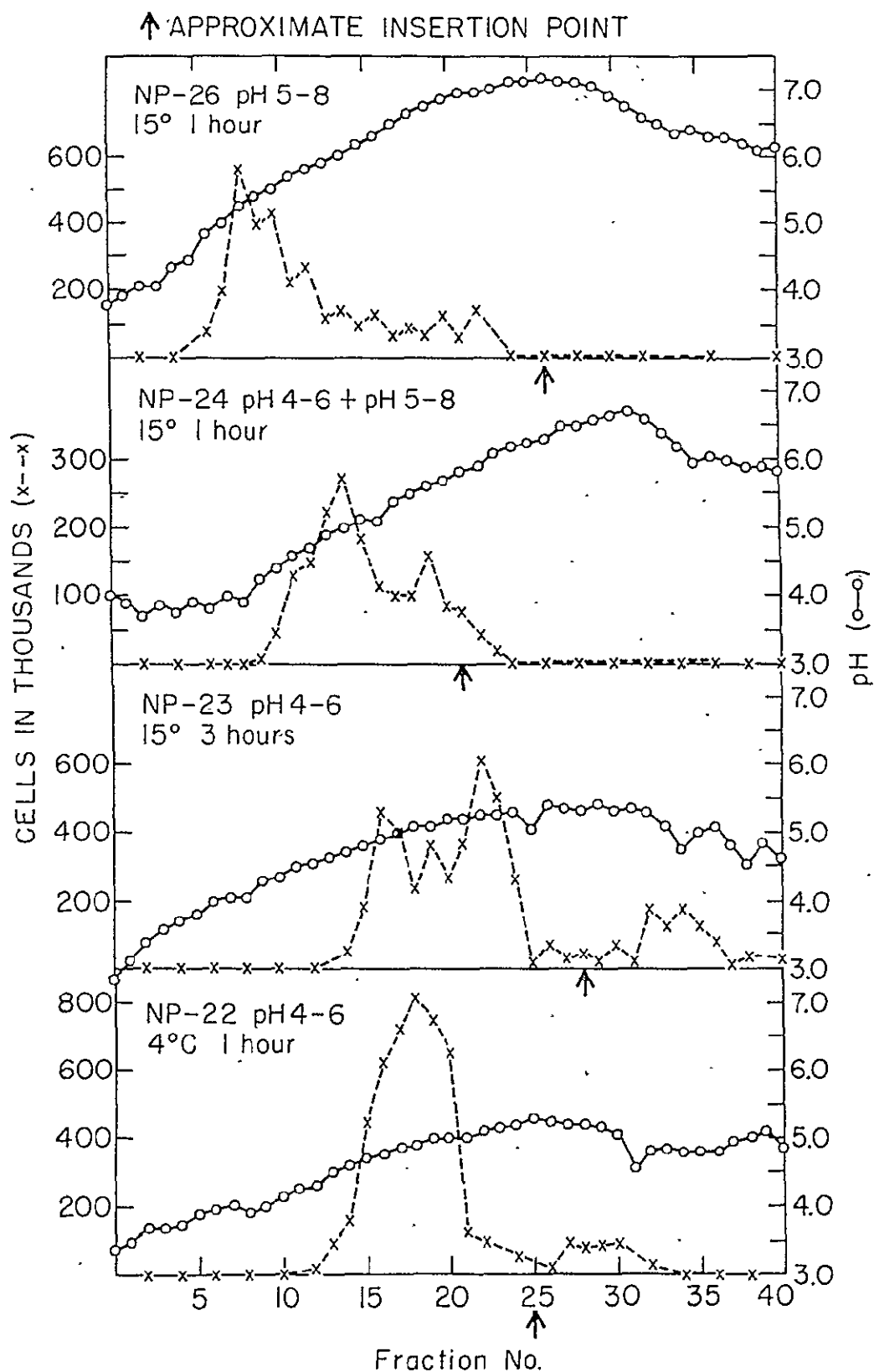
Figure 10

TABLE 1

Exp. #	Temp (°C)	Time (min.)	Ampholine pH range	%	# cells loaded (x10^6)	% cells Recovered*	% cells Recovered from buffer**	Insertion pH	Ficoll Range (% low	hi
NP-27	15	75	4-6	0.75	14.9	14	53	5.2	3	
			5-8	0.25						
NP-51	15	90	4-6	0.75	9.3	48	39	5.0	3	1
			5-8	0.25						
NP-47	15	150	4-6	0.75	8.3	86	74	6.2	8	1
			5-8	0.25						
NP-68	4	17.5 hrs	4-6	0.75	9.2	42	85	4.3	7	3
			5-8	0.25						
NP-75	15	17 hrs	4-6	0.75	7.1	32	97	5.2	8	2
			5-8	0.25						

*Percentage of cells recovered from electrophoresis column.

**Percentage of cells recovered from electrophoresis column buffer. These cells were not electrophoresed, but were maintained in the electrophoresis buffer at the insertion pH under conditions identical (i.e., in terms of temperature and time) to those being electrophoresed.

†Ficoll values represent concentration extremes to which cells were exposed with each experiment.

Figure 11

† APPROXIMATE INSERTION POINT
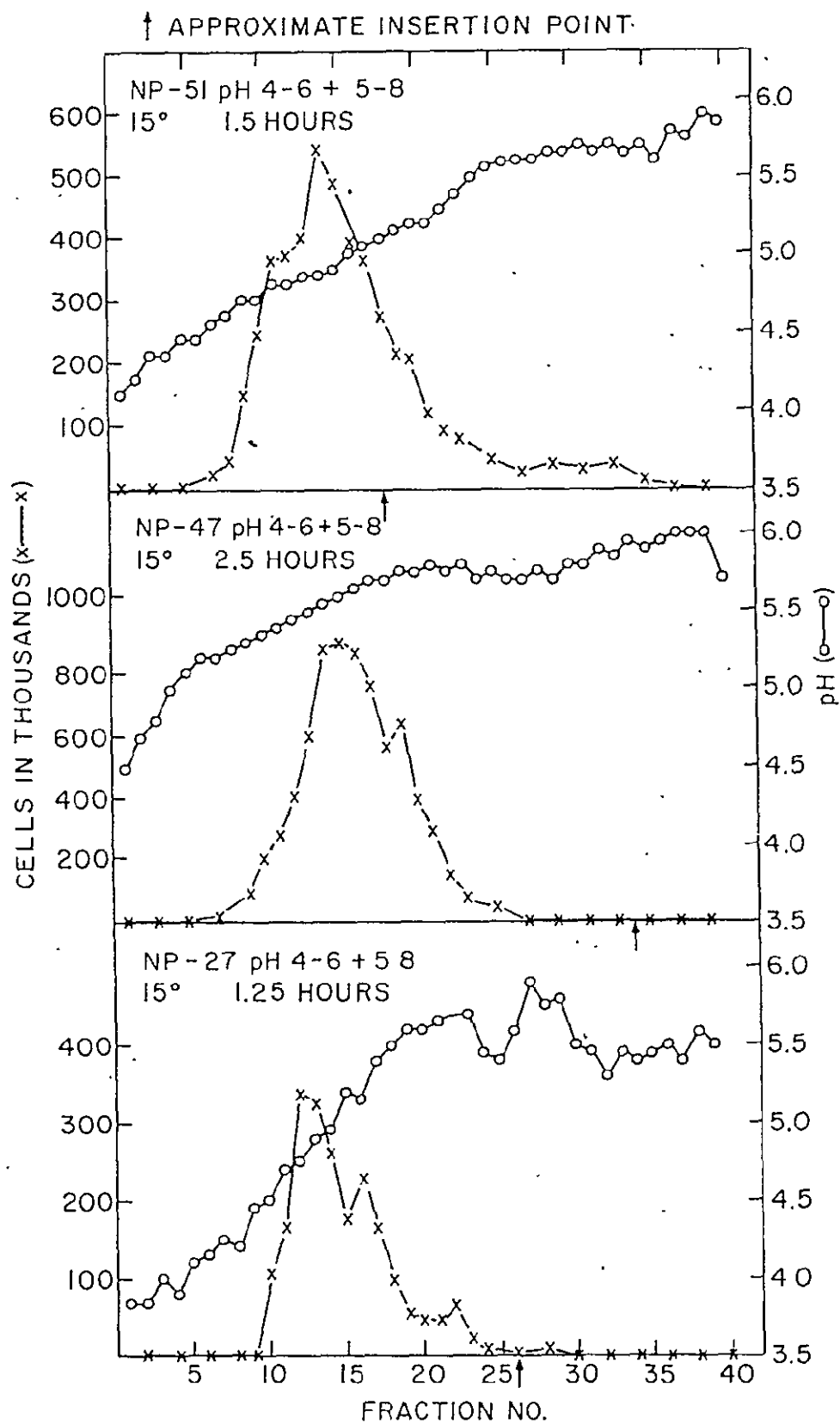
separated cells from these experiments usually showed poor staining with evidence of cell damage.

The reader will have noticed that the concentration of ficoll that the cells were exposed to in experiment NP47 was higher than either NP27 or 51 (see Table 1). Since the histology of the separated cells was poor, we had decided to increase the ficoll concentration in an effort to protect the cells. That this approach may be a useful one is suggested from the results of another experiment in which pituitary cells were suspended in different concentrations of ficoll (0-30%) for different times (0-21 hrs) kept at 4° C prior to staining. No electrophoresis was done on these cells. Recoveries of rat pituitary cells kept in these different ficoll solutions for various times are shown in Fig. 12. In general, recoveries seemed better as the ficoll concentration increased. Regardless of the concentration; however, 30% of the cells were lost at the 21 hour time point. Furthermore, cell doses seemed greater at earlier time points (0-3 hrs) in the 30% ficoll than when lower ficoll concentrations were used at comparable times (0-3 hrs). Detailed analysis of the cell types in these different preparations (Herlant's tetra-chrome stain) indicated, in general, that each type was present. This later result is important, for it shows that cell losses are probably not preferential.

In light of the results obtained with the higher ficoll concentrations (Fig. 12) and the relatively poor separations with the short (1.25-2.5 hr) times of electrophoresis (Fig. 11), we decided to run the isoelectric focusing columns in higher ficoll for longer periods of time. The results from two such runs are shown in Fig. 13. The results from these experiments indicated:

a. that the cell distributions were more spread out throughout the column; thereby apparently enhancing the chances for separation.

b. that the pH gradient was much more shallow than expected. We attribute this condition to the higher ficoll concentration.
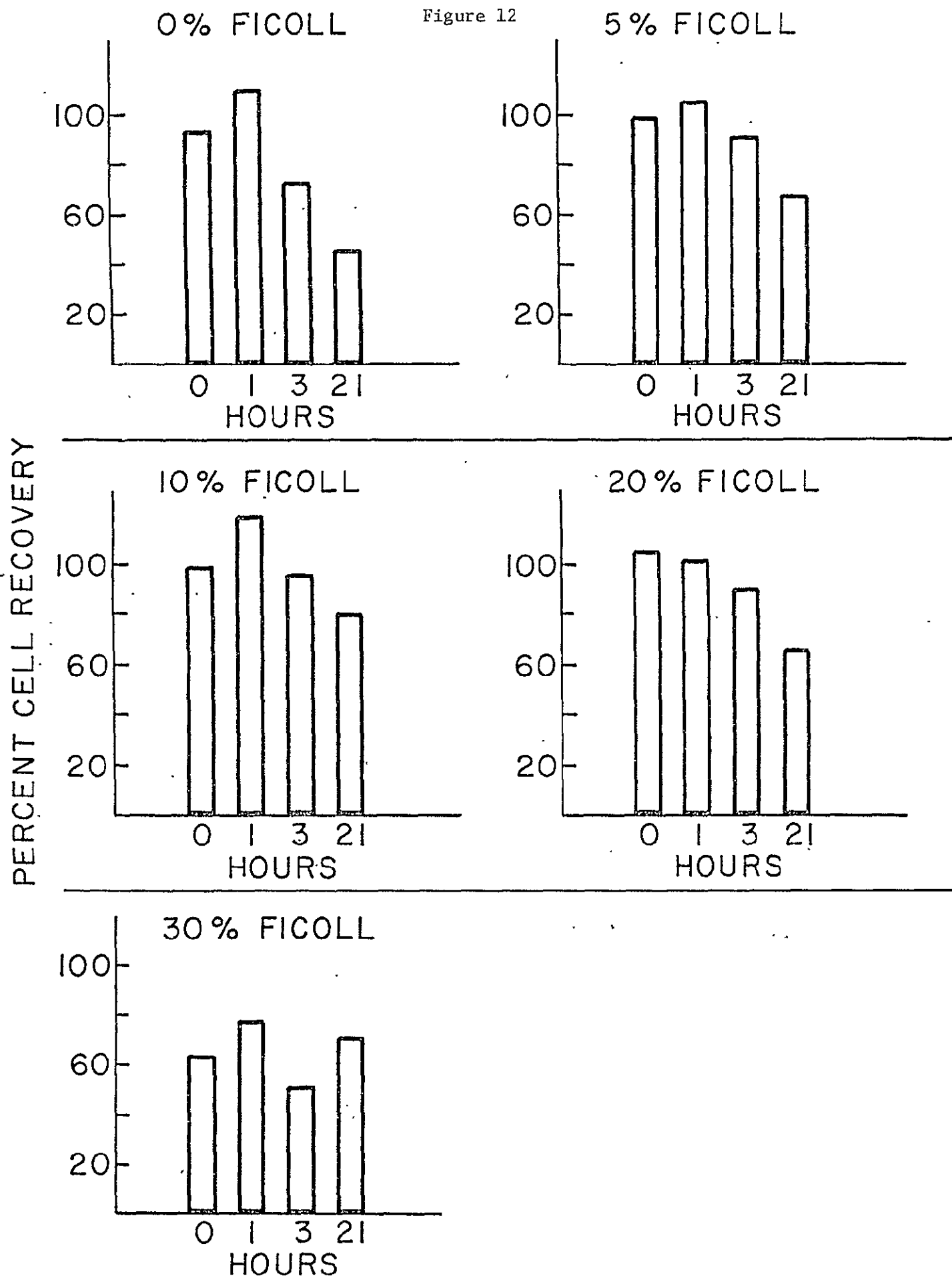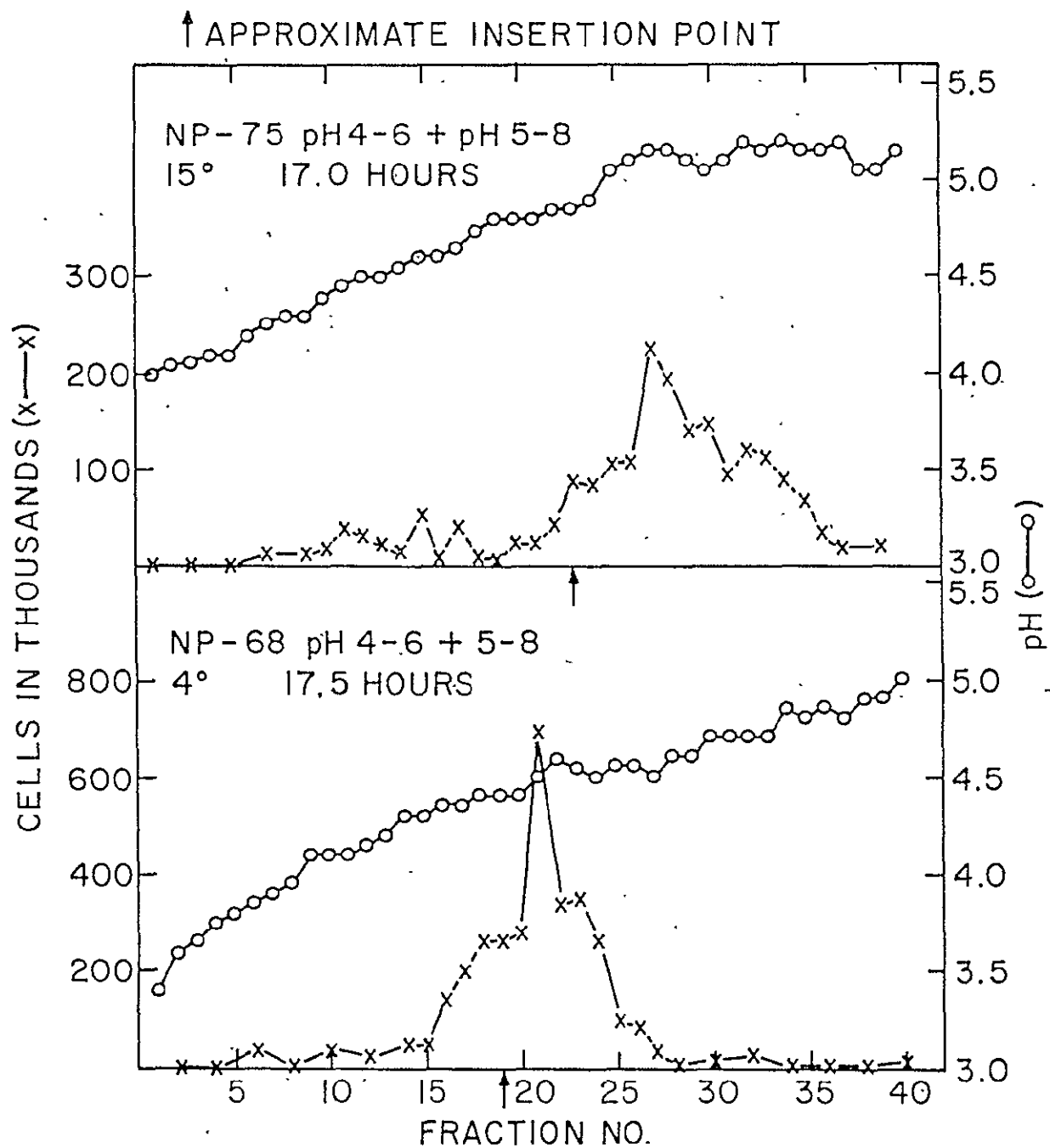
Figure 12

Figure 13

↑APPROXIMATE INSERTION POINT

NP-75 pH4-6 + pH5-8
15°   17.0 HOURS

NP-68 pH 4-6 + 5-8
4°   17.5 HOURS

CELLS IN THOUSANDS (x—x)

pH (o—o)

FRACTION NO.

We are currently attempting to remedy this condition by increasing

the ampholine concentration from 1% to 2.5%.

c. that pituitary cells recovered from these "long term" columns

tended to be clumped and

d. that suggestions of cell separation were beginning to be observed

(see slides enclosed with this report).

Finally, pooled fractions from experiment NP-75 (see Fig. 13) were

recently assayed for content of rat growth hormone by radioimmunoassay. The

following values were obtained:

Fraction #	ng rGH/200,000 cells
1-22	0
23-26	8,048
27-28	0
29-31	8,823
32-34	0
35-40	11,151

It should be pointed out that the 0 values (above) should not be taken

to indicate that no GH-cells were present. It is more likely that the extracts,

in these cases, were diluted too much. Given this reservation, these preliminary

data show a surprising spread of GH activity throughout the column. The higher

specific activity of the cells in fractions 35-40 argue 'for some kind of

separation taking place.

In summary, our work on the isoelectric focusing of pituitary cells during

the past year shows that the method offers promise in spite of drawbacks

relating to its difficulty in actual operation as well as their great number

of experimental variables (pH, ampholine and ficoll concentrations, time,

temp., etc.) to be considered for each experiment.

In the coming year, we intend to do several more experiments with this technique. In addition we will pursue experiments utilizing the simpler approach of density gradient electrophoresis.